

AMENDMENTS

In the Specification:

Please amend the first paragraph of the specification as follows:

D1 --This application is a Divisional of United States Patent Application Serial Number 09/422,073, filed October 21, 1999, now United States Patent Number 6,258,566, which is a Continuation of United States Patent Application Serial Number 08/989,332, filed December 11, 1997, now United States Patent Number 6,033,883, which claims the benefit under 35 U.S.C. §119 of the filing date of United States Provisional Application Serial Number 60/033,193, filed December 18, 1996, now abandoned. The contents of the provisional application are incorporated herein by reference.--

Please amend the paragraph starting at page 1, line 9 as follows:

D2 --The invention relates to production of polyketides in microbial hosts such as yeast and *E. coli* and to preparation of libraries containing a variety of functional polyketide synthases (PKSs) and the resulting variety of polyketides. More specifically, it concerns supplying portions of the polyketide synthase systems on separate vectors for simplicity in mixing and matching these portions to create a variety of PKS resultants polyketide synthases. This permits production of libraries of polyketide syntheses and polyketides through a combinatorial approach rather than manipulation focused on a single production system...--

Please amend the paragraph starting at page 2, line 6 as follows:

D3 --Polyketides generally are synthesized by condensation of two-carbon units in a manner analogous to fatty acid synthesis. In general, the synthesis involves a starter unit and extender units; these "two-carbon" units are derived from acylthioesters, typically acetyl, propionyl, malonyl or methylmalonyl coenzyme-A thioesters. There are two major classes of polyketide synthases (PKSs) which differ in the "manner" in which the catalytic sites are used -- the so-

D3 cont

called "aromatic" PKS and the modular PKS. The present invention employs coding sequences from both these classes as will further be explained in the herein application below.--

Please amend the paragraph starting at page 2, line 14 as follows:

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--Recombinant production of heterologous functional PKS -- i.e., a PKS which is capable of producing a polyketide -- has been achieved in *Streptomyces* and hybrid forms of aromatic PKSs have been produced in these hosts as well. See, for example, Khosla, C. *et al.* *J Bacteriol* (1993) 175:2194-2204; Hopwood, D.A. *et al.* *Nature* (1985) 314:642-644; Sherman, D.H. *et al.* *J Bacteriol* (1992) 174:6184-6190. In addition, recombinant production of modular PKS enzymes has been achieved in *Streptomyces* as described in PCT application WO 95/08548. In all of these cases, the PKS enzymes have been expressed from a single vector. A single vector which carried genes encoding PKS catalytic sites was transformed into *E. coli* by Roberts, G.A., *et al.*, *Eur J Biochem* (1993) 214:305-311, but the PKS was not functional, presumably due to lack of pantethenylation pantetheinylation of the acyl carrier proteins.--

Please amend the paragraph starting at page 2, line 25 as follows:

D5

--The present invention provides double or multivector systems for production of PKS and the resultant polyketides in a variety of hosts. The use of multiple vectors provides a means more efficiently to enhance the number of combinatorial forms of PKS and polyketides that can be prepared. Addition of the machinery for pantethenylation pantetheinylation of the acyl carrier proteins (i.e., a holo ACP synthase) permits production of polyketides in a wide spectrum of hosts.--

Please amend the paragraph starting at page 6, line 18 as follows:

D6

--On the other hand, the griseusin (gris) PKS contains five separate ORFs wherein the KS/AT, CLF, and ACP are on three ORFs, the KR is on a fourth, and the ARO is on a fifth.--

Please amend the paragraph starting at page 6, line 20 as follows:

--In the "modular" PKS systems, each catalytic site is used only once and the entire PKS is encoded as a series of "modules." Thus, the modular synthase protein contains a multiplicity of catalytic sites having the same type of catalytic activity. A minimal module contains at least a KS, an AT and an ACP. Optional additional activities include KR, DH, an enoylreductase (ER) and a thioesterase (TE) activity. Figure 2 shows, diagrammatically, the organization of the modular PKS system for the synthesis of the immediate precursor, 6-dEB, for the antibiotic erythromycin. As shown, there is a loading region followed by six modules; the thioesterase on module 6 effects release of the completed 6-deoxyerythronolide B (6-dEB) from the synthase to which it is coupled through a phosphopantetheinyl phosphopantetheinyl group. The diagram shows the progressive formation of the 6-deB which is cyclized after removal from the holo ACP on module 6 of the synthase. To convert 6-deB to erythromycin A, two sugar residues are added in subsequent reactions through the hydroxyl groups at positions 3 and 5--

Please amend the paragraph starting at page 7, line 21 as follows:

--Expression systems for the PKS proteins alone may not be sufficient for actual production of polyketides unless the recombinant host also contains holo ACP synthase activity which effects pantethoylation pantetheinylation of the acyl carrier protein. This activation step is necessary for the ability of the ACP to "pick up" the "2C" unit which is the starter unit or the growing polyketide chain in the series of Claisen condensations which result in the finished polyketide. For hosts lacking a phosphopantethoylating phosphopantetheinylating enzyme that behaves as a holo ACP synthase, the invention provides means for conferring this activity by supplying suitable expression systems for this enzyme. The expression system for the holo ACP synthase may be supplied on a vector separate from that carrying a PKS unit or may be supplied on the same vector or may be integrated into the chromosome of the host, or may be supplied as an expression system for a fusion protein with all or a portion of a polyketide synthase. In general, holo ACP synthases associated with fatty acid synthesis are not suitable; rather, synthases associated specifically with polyketide synthesis or with synthesis of nonribosomal proteins are useful in this regard.--

Please amend the paragraph starting at page 8, line 6 as follows:

D9

--Specifically, the modular and fungal PKS systems are not activated by phosphopantethenylation phosphopantetheinylation effected by the phosphopantethenylation phosphopantetheinylation enzymes indigenous endogenous to *E. coli*; however, enzymes derived from *Bacillus*, in particular the gramicidin holo ACP synthase of *Bacillus brevis* and the surfactin-related holo-ACP synthase from *Bacillus subtilis* can utilize the modular and fungal PKS ACP domains as substrates. As shown in the Examples below, while inclusion of an expression system for an appropriate holo-ACP synthase is not necessary for just the expression of the genes encoding fungal or modular PKS in *E. coli* or yeast, inclusion of such expression systems is required if polyketides are to be produced by the enzymes produced.--

Please amend the paragraph starting at page 9, line 21 as follows:

D10

--As stated above, in the present invention, the coding sequences for catalytic activities derived from the aromatic, fungal or modular PKS systems found in nature can be used in their native forms or modified by standard mutagenesis techniques to delete or diminish activity or to introduce an activity into a module in which it was not originally present. For example, a KR activity [[an]] can be introduced into a module normally lacking that function.--

Please amend the paragraph starting at page 14, line 11 as follows:

D11

--Another modification useful in varying the polyketides produced regardless of the host cell employed manipulates the PKS, in particular a modular or fungal PKS, to inactivate the ketosynthase (KS) on the first module. This permits enhanced efficiency in permitting the system to incorporate a suitable diketide thioester such as 3-hydroxy-2-methyl pantenoic pentanoic acid-N-acetyl cysteamine thioester, or similar thioesters of diketide analogs, as described by Jacobsen *et al. Science* (1997) 277:367-369. The construction of PKS modules containing inactivated ketosynthase regions is described in copending U.S. application 08/675,817, now U.S. Patent No. 6,080,555, and published in PCT application WO97/02358 incorporated herein by reference. These modified PKS modules can be employed in the various embodiments of the invention in preparing libraries using multivector methods and/or in *E. coli*

D11 cont
and yeast-based production organisms for the polyketides which may require the additional expression of a gene encoding a suitable holo-ACP synthase.--

Please amend the paragraph starting at page 16, line 21 as follows:

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--The *SacI/EcoRI* fragment containing the ADH2 promoter, the *EcoRI/Asp718* fragment containing the ADH2 terminator, and the *SacI/Asp718* fragment of pBlueScript were ligated to produce an intermediate vector, 43d2 which contains cloning sites (L2) for 6MSAS and the gene for the surfactant surfactin phosphopantethein phosphopantethein transferase from *B. subtilis* (the sfp gene). See Figure 6. It also contains sites (L1, L3) for transferring the promoter/terminator cassette into yeast shuttle vectors as well as sites (L1, L2) for moving the promoter/gene cassettes from the intermediate BlueScript vector into the yeast shuttle vector.--

Please amend the paragraph starting at page 18, line 9 as follows:

D13
--The *Bacillus subtilis* sfp gene encodes a holo ACP synthase, i.e., a phosphopantethenoyl phosphopantetheinyl transferase, and is inserted into plasmid YepFLAG-1 (IBI/Kodak).--

Please amend the paragraph starting at page 19, line 3 as follows:

D14
--Competent *Saccharomyces cerevesiae* InvSc1 cells were transformed with 102d (6 MSAS) and 128a (sfp holo ACP synthase). 128a was used in the first transformation with selection for tryptophan prototrophy; a successful transformed transformant was then transfected with 102d, with selection for tryptophan and uracil prototrophy. Transformants appeared after 48-72 hr at 30°C.--

Please amend the paragraph starting at page 20, line 1 as follows:

D15
--The kinetics for yeast growth and 6-MSA production for the transformant are shown in Figure 8A. As shown, the open squares represent growth as measured by OD₆₀₀. The closed circles represent the production of 6-MSA in g/L. The production of 6-MSA begins when

glucose is depleted consistent[[,]] with derepression of the ADH2 promoter. A plateau was reached after about 60 hr of growth and remained constant up to 150 hr.--

D15 cont.

Please amend the paragraph starting at page 20, line 19 as follows:

--The plasmid, 90, which contains a T5 promoter, 2 lac operators, and ~~lac^q-[2]~~ lac^d was constructed by ligating a 1.1-kbp *XhoI/XbaI* fragment of pQE60 (Qiagen) to the larger *XhoI/XbaI* fragment of pET22b(+) (Novagen). A *PstI/EcoRI* restriction fragment containing the DNA encoding module 6 KR-ACP-TE was ligated into plasmid 90 to give plasmid 104, an expression vector for this module.--

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Please amend page 21, line 2 as follows:

--Phosphopantethenylation Phosphopantetheinylation of Module 6 KR-ACP-TE--

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Please amend the paragraph starting at page 21, line 25 as follows:

--The module 6 KR-ACP-TE fragment of DEBS was efficiently labeled upon coexpression with GsP and with EntD, while no labeling was observed upon coexpression with ACPS. The inability of ACPS to activate the DEBS fragment is expected based on the known inactivity and lack of phosphopantethenylation phosphopantetheinylation of the DEBS protein when expressed in *E. coli* (Roberts *et al. Eur J Biochem* (1993) 214:305-311).--

D18

Please amend the paragraph starting at page 22, line 8 as follows:

--All assays were performed in 10 mM MgCl₂, 50 mM Tris-HCl (pH 8.8), in a total volume of 100 uL, and contained 40,000 cpm of 3H-coenzyme A and 0.39 uM sfp. A positive control contained 1.8 uM PheAT domain from gramicidin synthetase (Dr. Daniel Santi, UCSF) which is normally pantethenoylated pantetheinylated by sfp. Reactions were kept 12 h at 37°C, then boiled in SDS gel loading buffer and analyzed on a 10% SDS-PAGE gel. The gel was stained with Coomassie Blue, photographed, soaked in Amplify (Amersham), dried, and autoradiographed using Kodak Bio-MAX film for 2 days.--

Please amend the paragraph starting at page 23, line 8 as follows:

D²⁰

--The concentration of 6-MSA was estimated as described in Example 4 from a plot of concentration vs integrated [[are a]] area of corresponding HPLC peak using an authentic sample. The identity of the product was confirmed by LC-mass spectroscopy, which revealed [M+H]⁺ = 153, with a major fragment at m/z = 135 corresponding to loss of H₂O. Under these conditions, the culture produced 50 mg/L of 6-methylsalicylic acid.--

Please amend the paragraph starting at page 23, line 13 as follows:

D²¹

--The production of 6-MSA in *E. coli* was dependent on the presence of the plasmid encoding the sfp protein. *E. coli* transformed with only the 6-MSAS expression vector, 109, when induced by IPTG followed by incubation at 37°C for 4 hr, showed production of the approximately 190 kD 6-MSAS at about 5% of total protein. However, most of the protein was insoluble and 6-MSA was not detected in the medium. When the β-alanine auxotroph *E. coli* SJ16 containing the 6-MSAS expression vector 109 was incubated with labeled β-alanine before and after induction, no radioactivity was found in the 6-MSAS band on SDS-PAGE; thus, it appears the 6-MSAS was not modified with the phosphopantetheinyl phosphopantetheinyl cofactor by endogenous transferase. In a similar experiment involving *E. coli* SJ16 cotransformed with both plasmid 108 and 109, a detectable amount of radioactivity was found in the 190 kD 6-MSAS band; however, no 6-MSA was detected under these conditions. However, when the temperature of incubation was lowered to promote proper protein folding and glycerol was added to the medium to increase levels of intracellular malonyl CoA substrate, production of 6-MSA was improved. Thus, when cells were grown at 30°C in the absence of glycerol or at 37°C in the presence of 10% glycerol, no 6-MSA was produced. However, when grown as described above at 30°C in the presence of 10% glycerol, 6-MSA was produced up to about 75 mg/L after 24 hr of incubation. The kinetics of production are shown in Figure 8B.--

Please amend the paragraph starting at page 24, line 7 as follows:

D 22 --A fusion protein between the *Penicillium patulum* 6-methylsalicylic acid synthase (6-MSAS) and the *Bacillus subtilis* surfactin holo ACP synthase (sfp) [[was]] is made as follows:--

Please amend the paragraph starting at page 24, line 10 as follows:

D 23 --A 5.3-kbp *NdeI/HindIII* fragment containing the 6-MSAS gene (see Example 1) [[was]] is ligated with a 708-bp *HindIII/XbaI* fragment containing the sfp gene (see Example 3) and with *NdeI/XbaI*-restricted 43d2 (see Example 1) to produce intermediate plasmid 69. A ca. 6-kbp *NotI/RsrII* restriction fragment from 69 [[was]] is ligated with *NotI/RsrII*-restricted 101c (see Example 1) to yield the yeast expression vector 26a1 (see Example 1). This vector contains the 6-MSAS/sfp fusion gene between the ADH2 promoter/terminator pair.--

Please amend the paragraph starting at page 24, line 17 as follows:

D 24 --The resulting fusion protein consisted consists of connecting the C-terminal lysine of 6-MSAS with the N-terminal methionine of sfp using an (alanine)₃ linker, such that the DNA sequence of the gene in the region of the fusion [[was]] is:

5'-AAGCTTGCCAAA-GCCGCCGCC-ATGAAGATTAC-3'

where the lysine and methionine codons are underlined.--

Please amend the paragraph starting at page 24, line 22 as follows:

D 25 --Transformation of *S. cerevesiae* InvSc1 with 26a1 and culturing as described in Example 3 resulted results in production of 6-methylsalicylic acid at a level comparable with that resulting from expression of 6-MSAS and sfp as separate genes. The fusion protein thus combines the enzymatic activities of 6-MSAS and of sfp, self phosphopantethoylates phosphopantetheinylates, and produces polyketide product.--

Please amend the paragraph starting at page 25, line 14 as follows:

D 26 --A. The eryAIII gene (encoding modules 5 and 6 and the thioesterase of DEBS) under control of the actinorhodin promoter was cloned into pSET152. The resulting vector was used to

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cont

transform *S. lividans* K4-114, a strain in which the actinorhodin gene has been deleted by homologous recombination by standard methods (US patent application 08/238,811, now U.S. Patent No. 5,672,491, incorporated herein by reference). Apramycin-resistant transformants were selected.--

Please amend the paragraph starting at page 26, line 20 as follows:

D27

--A method by which the entire actinorhodin gene cluster is removed from these organisms and replaced with an antibiotic marker through homologous recombination has been described (US patent application 08/238,811, now U.S. Patent No. 5,672,491). This method is adapted as follows: The recombination vector consists of any vector capable of generating single-stranded DNA (e.g., pBlueScript) containing the following elements: 1) a DNA sequence homologous to the 5' 1-kbp end of the act cluster; 2) a resistance marker (e.g., hygromycin or thiostrepton); 3) the act II-orf4 activator gene; 4) the act promoter; 5) one or more genes of the foreign PKS; and 6) a DNA sequence homologous to the 3' 1-kbp end of the act cluster. Transformation of *S. coelicolor* or *S. lividans* with the recombination vector followed by selection for hygromycin resistance and screening for loss of blue color provides a host lacking the actinorhodin gene cluster and containing a chromosomal copy of the foreign PKS genes along with the needed actinorhodin control elements. This host is subsequently transformed by replicating vectors (e.g., SCP2*-based plasmids) and/or with integrating phage vectors (e.g., pSET152) containing other genes of the foreign PKS to complete the set of PKS genes and produce polyketide product.--